REMARKS

This Amendment responds to the Office Action mailed July 23, 2009. With this amendment, Applicants amend claim 1, and cancel claim 4. Applicants note that the Office has deemed claims 5-13 as being directed to non-elected subject matter and therefore withdrawn these claims from consideration. No new matter has been added with the present amendment. Support for the amendment can be found throughout the specification and claims as filed. Claims 1 and 5-13 are pending.

Formalities

Applicants note with appreciation that the Office has withdrawn prior rejections of the claims under 35 U.S.C. § § 101 and 112, second paragraph, for indefiniteness and lack of written description. The Applicants also note with appreciation the withdrawal-in-part of the rejections under 35 U.S.C. § 112, first paragraph.

Claim Rejections – 35 U.S.C. § 112, First Paragraph – Enablement

The Action maintains the rejections to claims 1 and 4 under 35 U.S.C. § 112, first paragraph, as allegedly failing to comply with the enablement requirement. The reasons were set forth in the previous Office Action. Briefly, the rejection turns on the Action's conclusions that (1) there is not a reliable and robust relationship between the required nucleotide content and the risk of arteriosclerotic disease including myocardial infarction, and (2) that the present invention

is unpredictable as to whether the asserted association of the instant specification would in fact reliably or robustly be reproduced in any other different population outside of a Japanese population. Applicants respectfully disagree with the Action's conclusions.

Applicants initially note that claim 1 has been amended to recite myocardial infarction. Claim 1 recites: "A method for determining an increased risk of myocardial infarction in humans, which comprises detecting in a biological sample obtained from a human subject, said sample comprising nucleic acids from the subject, a nucleotide at position 3279 of SEQ ID NO:1; wherein the presence of a C at position 3279 of SEQ ID NO:1 is indicative of an increased risk of myocardial infarction."

Applicants submit that the specification enables the present invention by providing ample direction and a detailed explanation of how to perform the invention. The working example, on page 20 of the specification, states that

> galectin-1 and galectin-2 were found to bind to LTA (lymphotoxin-α), and functional variations in these gene products were found to have led to functional variations in LTA, which could be associated with susceptibility to myocardial infarction. Accordingly, novel single nucleotide polymorphisms (SNPs) in these genes were identified and discovered, and the discovered SNPs were used to subject about 2300 patients and about 2300 controls to the case-control association study. As a result, it was found that the quantity of minor homozygotes (TT allele) of the novel SNPs (3279 C>T) in intron 1 of the galectin-2 gene was significantly small in myocardial infarction patients (X^2=25.3, P=0.0000005; odds ratio=1.6) (Table 1) (where the nucleotide number depends on the variant designation) (See pages 20-21 of the specification). This indicates that SNPs at nucleotide 3279 in intron 1 of galectin-2 are factors that act protectively in myocardial infarction and that functional variations in galectin-2 may be associated with myocardial infarction.

The specification further states that when the "nucleotide in position 3279 in the nucleotide sequence of intron 1 of the galectin-2 gene as shown in SEQ ID NO: 1 is C (3279C in intron 1 of galectin-2), for example, the expression level of galectin-2 can be determined to be low" (see specification pages 7-8). "On the other hand, when the nucleotide in position 3279 in the nucleotide sequence of intron 1 of the galectin-2 gene as shown in SEQ ID NO: 1 is T (3279T in intron 1 of galectin-2), it can be determined that inflammatory disease has not been developed or is less likely to be developed" (see specification pages 7-8).

Applicants further submit that one of ordinary skill in the art is enabled to make and use the present invention, in addition to through the specification's teachings, through the teachings of pre- and post-filing references. These references teach that the SNP at position 3279 of the galectin-2 gene can determine an increased risk of arteriosclerotic disease, and more specifically myocardial infarction.

For example, in Yamada et al. (International Journal of Molecular Medicine, (2008), Vol. 21, pages 801-808, hereinafter "Yamada")[1], the 3279C->T polymorphism of LGAL2 was found to be associated with a prevalence of arteriosclerotic disease and atherothrombotic cerebral infarction (see page 807, column 1). Applicants note that Ozaki et al. (Nature (2004), Vol. 429, pages 72 -75, hereinafter "Ozaki") [2] found that "the SNP (3279C->T) in intron-1 of LGALS2 was significantly associated with MI" (see page 72, column 1, and Supplementary Table 1). Furthermore, Ohnishi et al. (J. Jpn. Coll. Angiol., (2004), Vol. 44, pages 175-178, hereinafter "Ohnishi"), teaches that the SNP in LGALS2, which encodes galectin-2 and binds to lymphotoxin-alpha protein, "is significantly associated with susceptibility to MI" (see English

[1] Applicants note that copies of Yamada, Asselbergs, and Szolnoki are submitted herewith.
[2] Applicants note that Ozaki and Ohnishi have been submitted in the Information Disclosure Statement of January 18, 2008.

abstract). Additionally, Szolnoki et al. (Clinical Neurology and Neurosurgery (2009), Vol. 111, pages 227-230, hereinafter "Szolnoki"), teaches that the "LGALS2 3279TT homozygote variant has been demonstrated to exert protection against myocardial infarction by reducing the transcriptional level of galectin-2" (see abstract). Asselbergs et al. (Clinical Science (2007), Vol. 112, pages 291-298, hereinafter "Asselbergs"), a case-control study conducted on Americans, the C3279T polymorphism of the galectin-2 gene was shown to have a significant association with arteriosclerotic disease and coronary heart disease for American women (see page 295, columns 1 and 2).

Applicants note that Szolnoki teaches the association of the galectin-2 gene polymorphism in cerebral infarction in *non-Japanese* populations. Additionally, Asselbergs' study was on an American population.

Applicants recognize that Asselbergs reports a difference between men and women. However, Applicants note that Asselbergs shows a difference between the C reactive protein (CRP) levels of the male and female populations in the study (see Table 1). CRPs, which increase during systemic inflammation, indicate an inflammatory reaction in the body and are used as a myocardial infarction marker. Asselbergs' findings show that the degree of inflammatory reaction in the male patient population is lower than that of the female population. However, this is likely due to the fact that the male patient population in Asselbergs has a type of heart disease that is different from that of the female patient population. It might also be due to the fact that the male patient population had a mild (moderate) type of the disease. Applicants further note that in Asselbergs, the population is divided by gender, and the population may be hierarchized. All of these factors may account for the disparity in the findings of an association

between the polymorphism and the arteriosclerotic and coronary heart disease in the male and female populations.

Thus, contrary to the Office's assertions, the correlation between the polymorphism and arteriosclerotic disease, specifically myocardial infarction, extends to non-Japanese populations.

Applicants submit that it appears that the Office may not understand the essence of the present invention. The Action discusses the "unpredictability with regard to the association of any particular sequence with a particular phenotype" (Office Action, page 4). However the present invention does not determine that *a phenotype* (i.e., myocardial infarction) *is always observed* in a patient having a certain SNP at position 3279 of the galectin-2 gene. Rather, the present invention determines an *increased risk of a phenotype* (i.e., myocardial infarction). Applicants note that it may not always be possible to make a definitive diagnosis of a disease by detecting one SNP, but it may be possible to determine an increased risk of a disease by detecting one SNP.

Furthermore, in contrast to the Action's assertion, Applicants note that the present invention does not require the testing of a larger number of samples or undue experimentation. The Action alleges that a "large and prohibitive amount of experimentation would have to be performed in order to make and use the claimed invention" (Office Action, page 6). It appears that the Action confuses the case study analyses that teach the association of a particular SNP with a particular disease, with the present invention, which involves determining an increased risk of myocardial infarction of one person. Applicants note that the present invention was completed based on findings described in the specification. Still further, as explained above, several pre- and post-filing references demonstrate the association between the 3279C->T SNP

on the galectin-2 gene and arteriosclerotic diseases such as myocardial infarction. The present invention is *not* a case study analyzing the association of SNPs with particular disease. The present invention uses a sample of one person to determine the increased risk of myocardial infarction for that person. Applicants therefore submit that no large or prohibitive amount of experimentation would have to be performed in order to make and use the claimed invention. Either the correlation exists or it does not, and any amount of experimentation will not change that fact.

The Action also appears to mischaracterize the present invention. The Action contends that the "claims broadly recite detecting the presence or absence of a C at position 3279 of SEQ ID NO:1, and thus the claimed methods do not recite the detection of the recited nucleotide content" (Office Action, page 3). Applicants respectfully disagree with the Action's contention.

Applicants submit that the present invention in fact detects the nucleotide "content" at position 3279. If that content is "C," in other words the presence of a C is detected, then it is determined that there is an increased risk of myocardial infarction. However, if the content is "T," or in other words the absence of a C is detected, then there is no determination of an increased risk of myocardial infarction. Applicants submit that a clear understanding of the essence of the present invention and the claim language will allow the Office to appreciate that the specification enables one of ordinary skill in the art to make and use the present invention in its full scope, and does not require undue experimentation. While Applicants submit that this should be clear from the specification, Applicant's amend the claims in an attempt to render them even clearer.

In making the present rejection, the Action relies on post-filing references to support its assertion that the present invention is not predictable. However, in balancing the teachings of the references that the Action relies on with the references relied on by Applicants, the weight of the evidence supports Applicants' assertion that the specification enables the present invention, in its full scope.

In support of its arguments, the Action relies on Mangino et al. (Atherosclerosis (2007), Vol. 194, pages 112-115, hereinafter "Mangino") and Sedlacek et al. (J. Mol. Med. (2007), Vol. 85, page 997-1004, hereinafter "Sedlacek") to teach that there is no significant association between the 3279C->T SNP and myocardial infarction in the Caucasian population. The Action states that Applicants' arguments with regard to why Mangino and Sedlacek should be discounted were not persuasive.

Applicants respectfully note that in the previous response Applicants explained the association studies of Mangino and Sedlacek failed to take into account sampling bias with regard to the non-Japanese populations tested. Therefore, the findings of Mangino and Sedlacek appeared to be less reliable than association studies of non-Japanese populations that do take sampling bias into account.

An important aspect of association analysis is that the sample number of the association analysis is sufficiently large and that hierarchization of the sample (sampling bias) is not present. Non-Japanese populations are generally heterogeneous populations, and therefore are not suitable for association analysis in general. In particular, it is difficult to interpret the results which were obtained in non-Japanese people. Neither Mangino nor Sedlacek accounted for the sampling bias in their tested populations. Therefore, Applicants respectfully submit that the

teachings of Mangino and Sedlacek should not be relied upon to conclude that there is no association between the 3279C->T SNP and any arteriosclerotic disease, and myocardial infarction in particular.

The Action also relies on Kimura et al. (Tissue Antigens (2007), Vol. 69, pages. 265-269, hereinafter "Kimura") to teach that there is no association of the 3279C->T SNP with myocardial infarction in the Japanese and Korean populations tested. However, Applicants note that Kimura acknowledges possible reasons why the findings showed no association between the SNP and myocardial infarction. In particular, Kimura states that "we acknowledge that these findings require additional studies, and there is an apparent study limitation here that we did not strictly match the background of risk factor for MI in the patients and controls" (page 269, first column). The authors further noted that "the evaluation of coronary atherosclerosis (affected vessels) was not performed in the controls" (page 269, first column).

Applicants also note that the Action cites Lucenti (Scientist, Dec. 20, 2004, p. 20) and Hegele (Arterioscler. Throm. Vasc. Biol., 2002, Vol. 22, p. 1058), contending that gene association studies are unpredictable. However, Applicants note that although both references cite weaknesses in gene association studies, they also provide advice so that the weaknesses can be overcome. For example, Hegele provides a table of desirable attributes of genetic association studies, one desirable attribute being optimizing sampling (see page 1060). In Lucenti, a cited researcher suggests including a larger sample size and a greater number of family-based studies to avoid population stratification (see column 2, lines 16-24).

Optimization of sampling size and including a larger sample size and more family-based studies to avoid population stratification is also known as sampling bias or hierarchization.

Applicants have previously pointed out the sampling bias in references cited by the Action, in particular, Mangino and Sedlacek. Applicants submit that the references cited by the Office in support of its rejection, Hegele and Lucenti, actually support Applicants' contention that Mangino and Sedlacek should be discounted.

Applicants respectfully submit that the pre- and post-filing art supports the assertion that the specification enables the present invention, outweighing the support offered by the Office. Applicants note that in making the current rejection, the Office does not appear to properly consider the weight of Applicants' evidence.

Applicants submit that in balancing the evidence of both sides, the weight supports that the specification enables the claimed invention. Therefore, Applicants respectfully request the withdrawal of the 35 U.S.C. § 112, second paragraph rejection with regard to claim 1.

CONCLUSION

In view of the foregoing, the Examiner is respectfully requested to withdraw the rejections of record and allow all the pending claims.

Applicants invite the Examiner to contact the undersigned with any questions.

Respectfully Submitted,
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Effects of lymphotoxin-α gene and galectin-2 gene polymorphisms on inflammatory biomarkers, cellular adhesion molecules and risk of coronary heart disease

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ABSTRACT

The pro-inflammatory cytokine LTA (lymphotoxin-α) has multiple functions in regulating the immune system and may contribute to inflammatory processes leading to CHD (coronary heart disease). The aim of the present study was to investigate whether the common C804A (resulting in a $Thr^{26} \rightarrow$ Asp amino acid substitution) and A252G polymorphisms of the *LTA* gene and the C3279T polymorphism of the galectin-2 (*LGALS2*) gene, which affects LTA secretion, are associated with inflammatory parameters and cell adhesion molecules, and whether these polymorphisms are related to CHD in American women and men. We conducted a prospective nested case-control study within the Nurses' Health Study and Health Professionals Follow-Up Study. Among participants free of cardiovascular disease at baseline, 249 women and 266 men developed CHD during 8 and 6 years of follow-up respectively, and we matched controls 2:1 based on age and smoking. The *LGALS2* gene variant was significantly associated with a decreased risk of CHD in women [odds ratio (95% confidence interval), 0.70 (0.50–0.97); $P = 0.03$]. In addition, the *LGALS2* polymorphism was directly associated with CRP (C-reactive protein) levels in cases from both studies ($P < 0.05$). The *LTA* gene polymorphisms were directly associated with levels of sTNFRs (soluble tumour necrosis factor receptors) and VCAM-1 (vascular cell adhesion molecule-1) in both women and men with CHD ($P < 0.05$). However, no overall effect was demonstrated between *LTA* gene polymorphisms and risk of CHD.

Key words: coronary artery disease, cellular adhesion molecule, galectin-2, gene polymorphism, inflammation, lymphotoxin-α (LTA), myocardial infarction.

Abbreviations: BMI, body mass index; CABG, coronary artery bypass graft surgery; CHD, coronary heart disease; CI, confidence interval; CRP, C-reactive protein; HDL, high-density lipoprotein; HLA, human leucocyte antigen; HPFS, Health Professionals Follow-Up Study; IL-6, interleukin-6; LDL, low-density lipoprotein; LTA, lymphotoxin-α; NHS, Nurses' Health Study; PTCA, percutaneous transluminal coronary angioplasty; SNP, single nucleotide polymorphism; TNF, tumour necrosis factor; sTNF-R, soluble TNF receptor; VCAM-1, vascular cell adhesion molecule-1.

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INTRODUCTION

Inflammation plays an important role in the development and progression of atherosclerosis and is likely to play a critical role in the pathogenesis of plaque rupture which precedes a myocardial infarction [1]. The pro-inflammatory cytokine LTA [lymphotoxin-α; or TNF (tumour necrosis factor)-β] is found in atherosclerotic lesions [2] and may contribute to these processes. Furthermore, LTA may also induce adhesion molecules and cytokines from vascular endothelial and smooth muscle cells [3]. A large-scale association study from the Japanese Osaka Acute Coronary Insufficiency Study group identified functional SNPs (single nucleotide polymorphisms) within the *LTA* gene that were associated with a risk of myocardial infarction [*LTA* C804A (resulting in a $Thr^{26} \rightarrow Asp$ amino acid substitution) and *LTA* A252G] [4]. Among these SNPs, the *LTA* C804A polymorphism induced an almost 2-fold higher expression of E-selectin and VCAM-1 (vascular cell adhesion molecule-1) in cultured human coronary artery smooth muscle cells, and the presence of the *LTA* A252G gene polymorphism was associated with a 1.5-fold greater transcriptional activity of LTA [4]. The *LTA* and *TNF* (encoding TNF-α) genes are in significant linkage disequilibrium and are situated close to each other within the HLA (human leucocyte antigen) class III cluster on the short arm of chromosome 6.

Several other studies have examined the association between *LTA* gene polymorphisms and CHD (coronary heart disease), but these studies were based on identification of prevalent cases and the results were inconsistent [5–10]. In addition, genotype distribution in the original study by Ozaki et al. [4] deviated from Hardy–Weinberg equilibrium in the control group. A prospective longitudinal study is needed to investigate the association between *LTA* gene polymorphisms and CHD. Furthermore, the relationship between *LTA* gene polymorphisms and plasma levels of inflammatory markers and cell adhesion molecules are unknown.

Recently, the Japanese Osaka Acute Coronary Insufficiency Study group identified the galectin-2 protein as a regulator of LTA protein secretion and, therefore, also potentially important in modifying the degree of inflammation [3]. Both LTA and galectin-2 are expressed in smooth muscle cells and macrophages in the intima of atherosclerotic lesions of the coronary artery. Furthermore, the functional SNP (C3279T) in the galectin-2 (*LGALS2*) gene may be inversely associated with a risk of myocardial infarction [3]. No study has yet replicated these results in a prospective design.

We hypothesize that the common C804A and A252G polymorphisms of the *LTA* gene and the C3279T polymorphism of the *LGALS2* gene are associated with circulating inflammatory markers and cell adhesion molecules [i.e. CRP (C-reactive protein), IL-6 (interleukin-6), sTNF-R1 (soluble TNF receptor 1), sTNF-R2, VCAM-1 and E-selectin] and we aim to investigate whether these polymorphisms are related to CHD in a large nested case-control study among American women and men.

METHODS

Study population

We conducted a prospective nested case-control study within the NHS (Nurses' Health Study) and HPFS (Health Professionals Follow-Up Study). Among participants free of cardiovascular disease at baseline, 249 women and 266 men developed non-fatal myocardial infarction or fatal CHD during 8 and 6 years of follow-up respectively. As a secondary end point, we additionally identified 564 men who had CABG (coronary artery bypass graft surgery) or PTCA (percutaneous transluminal coronary angioplasty) during follow-up. Myocardial infarction was confirmed using World Health Organization criteria. Deaths were identified from State vital records and the National Death Index, or were reported by subjects' next of kin or the postal system. Fatal CHD was confirmed by hospital records or on autopsy, or if CHD was listed as the cause of death on the death certificate, if it was the underlying and most plausible cause, and if evidence of previous CHD was available. Confirmation of CABG/PTCA was based on self-reporting only. The study protocol was approved by the Institutional Review Board of the Brigham and Women's Hospital and the Harvard School of Public Health Human Subjects Committee Review Board; all participants provided informed consent.

Controls were selected 2:1 matched for age, smoking and month of blood draw. In addition, female controls were matched for fasting status. Biomarkers were measured for non-fatal myocardial infarction and fatal CHD cases and their controls only (the set of CABG/PTCA cases and controls did not have available plasma biomarkers).

Laboratory methods

CRP concentrations were determined using an immunoturbidimetric high-sensitivity assay (Denka Seiken) with day-to-day assay variability between 1 and 2%. Levels of IL-6, sTNF-R1, sTNF-R2, VCAM-1 and E-selectin were measured by ELISA (R&D Systems) [11], which have a day-to-day variability of 3.5–9.0%. HDL (high-density lipoprotein)-cholesterol and directly obtained LDL (low-density lipoprotein)-cholesterol were measured using standard methods with reagents from Roche Diagnostics and Genzyme.

Genotyping of polymorphisms

DNA was extracted from the buffy coat fraction of centrifuged blood using the QIAmp Blood Kit (Qiagen).

Table 1 Baseline characteristics of women and men who developed non-fatal myocardial infarction or fatal CHD during follow-up (cases) and matched event-free controls

Continuous variables are means ± S.E.M., except for CRP, IL-6 and E-selectin, which are medians (interquartile range). Men I, men who developed non-fatal myocardial infarction or fatal CHD during follow-up. MI, myocardial infarction.

	Women			Men I		
Variable	Cases ($n = 249$)	Controls ($n = 498$)	P value	Cases ($n = 266$)	Controls ($n = 522$)	P value
Age (years)	60.4 ± 0.4	60.3 ± 0.3	Matched	65.2 ± 8.3	65.1 ± 8.3	Matched
Current smoker (%)	32.1	31.9	Matched	12.0	12.1	Matched
Caucasians (%)	96.1	96.5	0.64	98.1	98.2	0.42
Diabetes (%)	19.7	6.6	< 0.001	9.4	4.5	0.007
History of hypertension (%)	57.4	29.3	< 0.001	42.1	30.9	0.002
BMI (kg/m^2)	26.8 ± 0.4	25.4 ± 0.2	< 0.001	26.2 ± 0.2	25.7 ± 0.2	0.06
Family history of MI (%)	27.7	12.3	< 0.001	51.5	37.3	< 0.001
HDL-cholesterol (mg/dl)	51.7 ± 0.9	60.3 ± 0.8	< 0.001	42.1 ± 0.7	45.9 ± 0.5	< 0.001
HDL-cholesterol (mmol/l)	1.32 ± 0.02	1.55 ± 0.02	–	1.08 ± 0.02	1.18 ± 0.01	–
LDL-cholesterol (mg/dl)	143.0 ± 2.26	132.3 ± 1.7	< 0.001	135.6 ± 2.2	126.8 ± 1.4	< 0.001
LDL-cholesterol (mmol/l)	3.67 ± 0.0	3.39 ± 0.04	–	3.48 ± 0.06	3.25 ± 0.04	–
CRP (mg/l)	3.12 (1.30–7.50)	2.20 (1.00–5.23)	< 0.001	1.68 (0.76–3.15)	1.08 (0.52–2.40)	< 0.001
IL-6 (pg/ml)	1.99 (1.30–3.04)	1.66 (1.16–2.67)	0.002	1.86 (1.10–3.07)	1.53 (0.97–2.88)	0.008
sTNF-R1 (pg/ml)	1447 ± 38	1270 ± 16	< 0.001	1514 ± 31	1504 ± 23	0.81
sTNF-R2 (pg/ml)	2790 ± 64	2491 ± 32	< 0.001	2992 ± 53	2943 ± 38	0.45
VCAM-1	726 ± 171	703 ± 157	0.13	1364 ± 331	1311 ± 318	0.03
E-selectin	49.4 (35.6–63.7)	44.1 (31.7–56.8)	0.001	–	–	–

We studied two SNPs in the *LTA* gene on chromosome 6p21 (HLA cluster): the *LTA* C804A (rs1041981) in exon 3 (resulting in the amino acid substitution $Thr^{26} \rightarrow Asn$) and *LTA* A252G (rs909253) in intron 1. In addition, we genotyped the C3279T (rs7291467) polymorphism in intron 1 of the *LGALS2* gene on chromosome 22q using Taqman SNP allelic discrimination by means of an ABI 7900HT (Applied Biosystems). Primer and probe sequences are available on request.

Statistical analysis

Continuous data are reported as means ± S.E.M. or medians (interquartile range) if the data were skewed. Categorical data are presented as per group percentages. Differences between subgroups were evaluated by Student's t test for the normally distributed continuous variables or by the Mann–Whitney test if data were skewed. Differences in genotype frequencies and other categorical data between cases and controls were compared with the χ^2 test or Fisher's exact test. Consistency of genotype frequencies with the Hardy–Weinberg equilibrium was tested using a χ^2 goodness-of-fit test on a contingency table of observed compared with expected genotype frequencies in cases and controls. Genotype–phenotype associations were examined with additive, dominant and recessive models using multivariate logistic regression analyses. Odds ratios for the occurrence of CHD and their 95% CIs (confidence intervals) were calculated after adjustment for matching factors. Linear mixed models were used to investigate the age-adjusted association between genotypes and inflammatory markers. In addition, linear mixed models were used to investigate the gene–environment interaction between BMI (body mass index) and *LTA* and *LGALS2* gene polymorphisms on inflammatory markers. All results were considered statistically significant if the two-sided P value for the test statistic was less than or equal to the set type I error rate (α) of 0.05. No adjustment for multiple comparisons was performed, because there were few statistical tests and there is good biological evidence that each of the biochemical systems being studied is functionally involved in regulating inflammatory status either directly or indirectly, suggesting the universal null hypothesis that is assumed for a Bonferroni-type correction does not apply to these data [12]. Analyses were performed using SAS version 9.1 (SAS Institute).

RESULTS

Baseline characteristics

The general characteristics of both the NHS as well as the HPFS, divided on the basis of cases and controls, are shown in Table 1. Cases were more likely to have diabetes, hypertension and a family history of myocardial infarction than matched controls. In addition, cases from both studies had significantly higher levels of LDL-cholesterol and lower levels of HDL-cholesterol.

Table 2 Genotype distributions among women who developed non-fatal myocardial infarction or fatal CHD during follow-up (cases) and matched event-free controls, and among men in groups I (Men I) and II (Men II) and matched event-free controls

Men I, men who developed non-fatal myocardial infarction or fatal CHD during follow-up; Men II, men in the Men I group ($n = 266$) plus men who underwent CABG or PTCA during follow-up ($n = 564$). *P* value for comparison between cases and controls.

	Women		Men I		Men II	
Genotype	Cases	Controls	Cases	Controls	Cases	Controls
LTA C804A (*n*)						
AA	101 (43.9 %)	208 (44.2 %)	118 (47.8 %)	221 (44.5 %)	384 (49.9 %)	720 (46.2 %)
CA	95 (41.3 %)	213 (45.2 %)	100 (40.5 %)	223 (44.9 %)	304 (39.5 %)	691 (44.4 %)
CC	34 (14.8 %)	50 (10.6 %)	29 (11.7 %)	53 (10.7 %)	81 (10.5 %)	145 (9.3 %)
P value	0.42		0.67		0.39	
LTA A252G (*n*)						
AA	103 (43.5 %)	207 (44.9 %)	119 (46.5 %)	223 (44.4 %)	389 (49.2 %)	730 (46.0 %)
AG	98 (41.4 %)	199 (43.2 %)	106 (41.4 %)	225 (44.8 %)	320 (40.5 %)	708 (44.6 %)
GG	36 (15.2 %)	55 (11.9 %)	31 (12.1 %)	54 (10.8 %)	82 (10.4 %)	148 (9.3 %)
P value	0.39		0.89		0.46	
LGALS2 C3279T (*n*)						
CC	102 (43.8 %)	162 (34.8 %)	72 (28.6 %)	170 (33.9 %)	230 (29.5 %)	506 (32.2 %)
CT	99 (42.5 %)	220 (47.3 %)	133 (52.8 %)	232 (46.2 %)	394 (50.5 %)	749 (47.6 %)
TT	32 (13.7 %)	83 (17.9 %)	47 (18.7 %)	100 (19.9 %)	157 (20.1 %)	319 (20.3 %)
P value	0.02		0.46		0.41	

Women with CHD had a higher BMI and higher levels of inflammatory markers, including CRP, IL-6, sTNF-R1, sTNF-R2 and E-selectin, than the matched control group. Men with CHD had significantly higher levels of CRP, IL-6 and VCAM-1, but mean sTNF-R1 and sTNF-R2 levels were not different between the groups. The characteristics did not change substantially when including men and matched controls who needed cardiac revascularization, the secondary end point (Men II). The distributions of genotypes were in Hardy–Weinberg equilibrium both in cases as well as controls ($P > 0.10$). The genotype frequencies are shown in Table 2. Only the distribution of the *LGALS2* gene polymorphism was significantly different between female cases compared with matched event-free controls. The pairwise linkage disequilibrium (D') and the correlation coefficient between *LTA* C804A and *LTA* A252G were 0.99. No correlation was present between the *LTA* gene polymorphisms and *LGALS2* gene polymorphism.

Association between *LTA* and *LGALS2* gene polymorphisms and markers of inflammation and cell adhesion molecules

Tables 3–5 show the age-adjusted levels of the inflammatory markers and cell adhesion molecules (i.e. CRP, IL-6, sTNF-R1, sTNF-R2, VCAM-1 and E-selectin) among the different genotypes. The *LTA* C804A polymorphism was associated with plasma levels of sTNF-R2 and VCAM-1 in both female and male cases. In addition, the *LTA* C804A gene polymorphism was significantly associated with IL-6 in men without CHD (Table 3). Similar results were found for the *LTA* A252G gene polymorphism, which was also associated with sTNF-R1 levels in both women as well as men with CHD (Table 4).The *LGALS2* gene polymorphism was associated with CRP levels in both male and female cases (Table 5). Furthermore, no significant interaction was present between BMI and the gene polymorphisms on the inflammatory markers in cases or controls from both the female and male cohorts.

Association between *LTA* and *LGALS2* gene polymorphisms and risk of CHD

Table 6 shows the results from unconditional multivariate logistic regression analyses for CHD. The *LGALS2* gene variant was inversely associated with a risk of CHD in women [odds ratio (95 % CI), 0.70 (0.50–0.97); $P = 0.03$]. This effect was independent of cardiovascular risk factors predictive of cardiovascular disease (diabetes, history of hypertension, BMI, family history of myocardial infarction, HDL-cholesterol, LDL-cholesterol, CRP, IL-6, sTNF-R1, sTNF-R2, VCAM-1 and E-selectin). The odds ratio (95 % CI) for CHD in women after adjustment for all these factors was 0.36 (0.22–0.59) ($P < 0.001$). This association was not present in men. After pooling the data from both women and men, we found a significant gender interaction between the *LGALS2* gene polymorphism and risk of CHD ($P = 0.01$ for interaction).

Table 3 Biomarker levels adjusted for age according to *LTA* C804A (rs1041981) genotype among women and men

Variables are means ± S.E.M., except for CRP, IL-6 and E-selectin, which are geometric means (95 % CIs). *Additive model, $P = 0.011$; dominant model, $P = 0.005$; recessive model, $P = 0.860$. †Additive model, $P = 0.029$; dominant model, $P = 0.952$; recessive model, $P = 0.009$. ‡Additive model, $P = 0.018$; dominant model, $P = 0.050$; recessive model, $P = 0.728$. §Additive model, $P = 0.194$; dominant model, $P = 0.471$; recessive model, $P = 0.076$. ¶Additive model, $P = 0.103$; dominant model, $P = 0.847$; recessive model, $P = 0.038$. ||Additive model, $P = 0.102$; dominant model, $P = 0.865$; recessive model, $P = 0.042$. Men I, men who developed non-fatal myocardial infarction or fatal CHD during follow-up.

(a) Cases

	Women			Men I		
Variable Genotype . . .	AA	CA	CC	AA	CA	CC
CRP	2.73 (2.21–3.36)	3.46 (2.70–4.43)	3.40 (2.31–5.01)	1.59 (1.30–1.94)	1.66 (1.39–1.99)	1.61 (1.05–2.47)
IL-6	1.99 (1.77–2.23)	2.15 (1.86–2.48)	1.92 (1.50–2.46)	2.36 (1.94–2.88)	2.07 (1.77–2.43)	2.09 (1.51–2.89)
sTNF-RI	1358 ± 45	1509 ± 66	1417 ± 98	1509 ± 46	1485 ± 40	1624 ± 92
sTNF-R2	2568 ± 74	2960 ± 106	2726 ± 151*	2968 ± 73	2930 ± 77	3400 ± 161†
VCAM-1	694 ± 17	769 ± 680	680 ± 39‡	1346 ± 25	1352 ± 39	1456 ± 56§
E-selectin	46.6 (42.6–51.1)	49.8 (45.7–54.3)	43.6 (38.2–49.7)	–	–	–

(b) Controls

	Women			Men I		
Variable Genotype . . .	AA	CA	CC	AA	CA	CC
CRP	2.18 (1.90–2.51)	2.27 (1.92–2.69)	2.01 (1.48–2.73)	1.18 (1.01–1.38)	1.23 (1.05–1.44)	0.96 (0.07–1.28)
IL-6	1.75 (1.59–1.92)	1.90 (1.73–2.08)	1.66 (1.39–1.99)	1.83 (1.60–2.09)	1.88 (1.68–2.10)	1.50 (1.24–1.80)¶
sTNF-RI	1272 ± 24	1272 ± 23	1215 ± 52	1476 ± 32	1539 ± 36	1466 ± 58
sTNF-R2	2493 ± 45	2503 ± 49	2426 ± 100	2931 ± 54	2953 ± 51	2816 ± 118
VCAM-1	715 ± 12	694 ± 13	695 ± 29	1303 ± 19	1325 ± 22	1234 ± 37‖
E-selectin	41.4 (39.1–43.9)	43.8 (41.4–46.3)	42.5 (37.6–48.1)	–	–	–

DISCUSSION

In the present large prospective nested case-control study among American women and men, we investigated the relationship between *LTA* and *LGALS2* gene polymorphisms and levels of inflammatory markers, cell adhesion molecules and risk of CHD. This study showed significant associations between the polymorphisms in the *LTA* and *LGALS2* genes and markers of inflammation and cell adhesion molecules, but no association was found between *LTA* gene polymorphisms and risk of CHD in women and men. For the *LGALS2* gene polymorphism, we found evidence of a significant gender interaction, with a significant association for women, but not men, with the risk of CHD.

Previous case-control and cross-sectional studies have examined the association between *LTA* gene polymorphisms and cardiovascular disease, but the results are inconsistent. The first study by Ozaki et al. [4] described significant associations between *LTA* gene polymorphisms and myocardial infarction; however, the authors did not adjust for relevant covariates, including gender and age, and the genotype distributions among the control subjects were not in Hardy–Weinberg equilibrium. The association between the *LTA* gene polymorphisms and CHD was confirmed in another Japanese population [5] and in the family-based European PROCARDIS (precocious coronary artery disease) study [6]. Furthermore, a significant association was found between the *LTA* C804A genotype and the extent of coronary atherosclerosis in Caucasian patients with angiographically confirmed coronary atherosclerosis [7]. In concordance with the present results, several other studies did not detect an association between *LTA* gene polymorphisms and myocardial infarction [8–10], and our findings are in agreement with a recent meta-analysis performed by Clarke et al. [10], which showed no relationship between *LTA* gene polymorphisms and CHD. In contrast with the previous reports included in this meta-analysis, we used unrelated controls selected from the same population as the cases.

Our present study has shown a significant association between the *LGALS2* gene polymorphism and reduced risk for CHD in women; however, this association could not be replicated in our male population. This statistical gender interaction might be a true biological interaction or may reflect differences in cardiovascular risk factors in the male and female study populations. The present study is in concordance with the findings of Ozaki et al. [3], who reported an association between the *LGALS2* gene polymorphism and myocardial infarction. However, their study did not provide any information about gender differences. Other functional studies published so far do not report differences in LTA secretion between genders,

Table 4 Biomarker levels adjusted for age according to *LTA* A252G (rs909253) genotype among women and men

Variables are means ± S.E.M., except for CRP, IL-6 and E-selectin, which are geometric means (95 % CIs). *Additive model, $P = 0.028$; dominant model, $P = 0.014$; recessive model, $P = 0.676$. †Additive model, $P = 0.002$; dominant model, $P = 0.001$; recessive model, $P = 0.606$. ‡Additive model, $P = 0.056$; dominant model, $P = 0.384$; recessive model, $P = 0.016$. §Additive model, $P = 0.009$; dominant model, $P = 0.017$; recessive model, $P = 0.342$. ¶Additive model, $P = 0.128$; dominant model, $P = 0.379$; recessive model, $P = 0.049$. ||Additive model, $P = 0.111$; dominant model, $P = 0.872$; recessive model, $P = 0.042$. Men 1, men who developed non-fatal myocardial infarction or fatal CHD during follow-up.

(a) Cases

	Women			Men 1		
Variable Genotype . . .	AA	AG	GG	AA	AG	GG
CRP	2.63 (2.12–3.26)	3.62 (2.84–4.62)	3.22 (2.17–4.77)	1.62 (1.33–1.98)	1.64 (1.38–1.94)	1.49 (0.99–2.24)
IL-6	1.97 (1.75–2.20)	2.17 (1.90–2.48)	1.87 (1.45–2.40)	2.27 (1.87–2.76)	2.08 (1.77–2.44)	1.96 (1.43–2.67)
sTNF-R1	1344 ± 44	1562 ± 68	1407 ± 93*	1493 ± 43	1497 ± 39	1604 ± 87
sTNF-R2	2553 ± 72	3051 ± 117	2711 ± 144†	2933 ± 73	2929 ± 74	3332 ± 157‡
VCAM-1	694 ± 17	776 ± 21	696 ± 40§	1340 ± 25	1350 ± 36	1457 ± 53¶
E-selectin	46.3 (42.5–50.5)	50.0 (45.8–54.6)	43.6 (38.4–49.4)	–	–	–

(b) Controls

	Women			Men 1		
Variable Genotype . . .	AA	AG	GG	AA	AG	GG
CRP	2.17 (1.88–2.50)	2.35 (1.98–2.80)	1.92 (1.46–2.54)	1.19 (1.02–1.40)	1.22 (1.05–1.43)	0.95 (0.72–1.27)
IL-6	1.75 (1.59–1.93)	1.93 (1.76–2.13)	1.67 (1.41–1.99)	1.83 (1.61–2.09)	1.89 (1.69–2.10)	1.51 (1.26–1.81)‖
sTNF-R1	1268 ± 24	1277 ± 24	1261 ± 52	1481 ± 31	1535 ± 36	1479 ± 57
sTNF-R2	2492 ± 45	2503 ± 52	2553 ± 106	2950 ± 54	2956 ± 52	2895 ± 121
VCAM-1	715 ± 12	688 ± 13	717 ± 32	1307 ± 19	1328 ± 22	1255 ± 41
E-selectin	42.2 (39.7–44.8)	43.4 (40.9–46.0)	42.9 (37.8–48.7)	–	–	–

Table 5 Biomarker levels adjusted for age according to *LGALS2* C3279T (rs7291467) genotype among women and men

Variables are means ± S.E.M., except for CRP, IL-6 and E-selectin, which are geometric means (95 % CIs). *Additive model, $P = 0.035$; dominant model, $P = 0.041$; recessive model, $P = 0.507$. †Additive model, $P = 0.051$; dominant model, $P = 0.017$; recessive model, $P = 0.281$. Men 1, men who developed non-fatal myocardial infarction or fatal CHD during follow-up.

(a) Cases

	Women			Men 1		
Variable Genotype . . .	CC	CT	TT	CC	CT	TT
CRP	2.61 (2.06–3.29)	3.88 (3.15–4.78)	2.73 (1.80–4.14)*	1.26 (1.02–1.56)	1.71 (1.42–2.05)	1.84 (1.37–2.47)†
IL-6	2.09 (1.82–2.40)	2.14 (1.90–2.40)	1.97 (1.49–2.60)	1.81 (1.54–2.12)	2.36 (1.97–2.82)	2.01 (1.55–2.59)
sTNF-R1	1426 ± 58	1447 ± 60	1468 ± 103	1478 ± 51	1490 ± 38	1557 ± 63
sTNF-R2	2765 ± 105	2802 ± 91	2847 ± 177	3017 ± 94	2920 ± 70	3071 ± 94
VCAM-1	721 ± 20	736 ± 22	702 ± 31	1412 ± 36	1329 ± 26	1392 ± 57
E-selectin	46.1 (42.3–50.1)	48.8 (44.5–53.4)	51.0 (44.1–59.1)	–	–	–

(b) Controls

	Women			Men 1		
Variable Genotype . . .	CC	CT	TT	CC	CT	TT
CRP	2.12 (1.80–2.49)	2.15 (1.83–2.54)	2.35 (1.87–2.94)	1.27 (1.06–1.52)	1.16 (1.01–1.34)	1.03 (0.80–1.34)
IL-6	1.79 (1.63–1.98)	1.85 (1.67–2.04)	1.75 (1.52–2.01)	1.92 (1.68–2.20)	1.70 (1.54–1.88)	1.94 (1.56–2.41)
sTNF-R1	1250 ± 25	1273 ± 24	1311 ± 38	1513 ± 34	1494 ± 34	1512 ± 56
sTNF-R2	2453 ± 50	2513 ± 50	2525 ± 67	3000 ± 64	2870 ± 51	3006 ± 82
VCAM-1	712 ± 16	698 ± 11	692 ± 23	1316 ± 27	1297 ± 19	1332 ± 26
E-selectin	43.2 (40.5–46.2)	41.5 (39.1–44.0)	46.5 (42.5–50.8)	–	–	–

Table 6 Associations of *LTA* and *LGALS2* gene polymorphisms with risk of CHF

Values are odds ratios (95 % CIs). Men I, men who developed non-fatal myocardial infarction or fatal CHD during follow-up; Men II, men in the Men I group ($n = 266$) plus men who underwent CABG or PTCA during follow-up ($n = 564$). P value for comparison between cases and controls. *$P = 0.0252$; †$P = 0.0312$.

	Women	Men I	Men II	Pooled (women and men I)
LTA C804A				
Additive	1.08 (0.85–1.36)	0.95 (0.76–1.20)	0.94 (0.82–1.07)	0.99 (0.84–1.17)
Dominant	0.98 (0.71–1.36)	0.87 (0.64–1.82)	0.86 (0.72–1.02)	0.92 (0.74–1.15)
Recessive	1.42 (0.88–2.28)	1.13 (0.70–1.84)	1.15 (0.86–1.54)	1.20 (0.86–1.67)
LTA A252G				
Additive	1.08 (0.86–1.36)	0.99 (0.79–1.24)	0.95 (0.83–1.08)	1.03 (0.87–1.20)
Dominant	1.04 (0.75–1.43)	0.92 (0.68–1.25)	0.88 (0.74–1.04)	0.96 (0.77–1.19)
Recessive	1.29 (0.81–2.05)	1.15 (0.72–1.85)	1.13 (0.85–1.50)	1.15 (0.84–1.59)
LGALS2 C3279T				
Additive	0.77 (0.61–0.97)*	1.09 (0.88–1.35)	1.05 (0.93–1.19)	0.94 (0.80–1.10)
Dominant	0.70 (0.50–0.97)†	1.29 (0.92–1.79)	1.14 (0.94–1.37)	0.94 (0.75–1.19)
Recessive	0.72 (0.46–1.13)	0.93 (0.63–1.37)	1.00 (0.80–1.23)	0.87 (0.64–1.17)

but, as shown in Table 1, levels of inflammatory markers differ between genders and, therefore, it is possible that LTA has a sex-specific range too. Future studies are needed to investigate whether LTA secretion differs between genders.

Surprisingly, no relationship was found between *LTA* gene polymorphisms and CRP. LTA is a pro-inflammatory cytokine acting through activation of NF-κB (nuclear factor κB), and previous reports have demonstrated a weak, but significant, association between an *LTA* gene polymorphism and CRP levels [10,13]. Galectin-2 has been shown [3] to affect LTA expression levels and might therefore influence CRP levels as well; however, the relationship between *LGALS2* genotype and CRP in the present study was opposite to that expected. We cannot exclude the role of chance or some counter-regulatory action which we did not capture with the genetic variation in *LGALS2*. Clearly, further study is needed to confirm or reject the present findings.

Interestingly, we found a significant association between *LTA* gene polymorphisms and the level of sTNF-R2 in both women and men. LTA is a pro-inflammatory cytokine that may contribute to atherosclerosis by activation of growth factors and cytokines, and by affecting the synthesis and stimulation of adhesion molecules [4]. LTA, like TNF-α, interacts with sTNF-R1 and sTNF-R2. sTNF-R concentrations are increased in patients with infectious diseases and may be useful as an indicator of LTA-induced inflammation [14]. On the other hand, the observed association between *LTA* gene polymorphisms and inflammatory markers might also represent an effect of the *TNF* gene or other genetic products of the HLA cluster, because the *LTA* gene is in significant linkage disequilibrium with the *TNF* gene located on chromosome 6 and the HLA cluster [15].

Furthermore, we detected a weak association between VCAM-1 and *LTA* gene polymorphisms; however, the direction of the associations between VCAM-1 and *LTA* gene polymorphisms found in the present study were not consistent among the cohorts. In contrast with the males, the variant genotype was associated with lower VCAM-1 levels in the female cases. This might be due to chance considering the borderline significance levels or indicate a true gender difference. Ozaki et al. [4] demonstrated previously that variant protein LTA 26A induced an increase in VCAM-1 and E-selectin in human coronary artery vascular smooth muscle cells. Elevated expression of adhesion molecules, such as VCAM-1 and E-selectin, might contribute to the pathogenesis of myocardial infarction, but despite the association between *LTA* gene polymorphisms and VCAM-1 no relationship between *LTA* gene polymorphisms and CHD could be demonstrated in the present study.

In conclusion, the present study has demonstrated an association between *LTA* and *LGALS2* gene polymorphisms and markers of inflammation and cell adhesion molecules, but did not detect a significant association between *LTA* gene polymorphisms and CHD in American women and men. Future studies are needed to replicate the observed association between the *LGALS2* gene polymorphism and reduced risk of CHD in women.

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Association of genetic variants with atherothrombotic cerebral infarction in Japanese individuals with metabolic syndrome

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Abstract. Metabolic syndrome is a risk factor for cardiovascular disease. The aim of the present study was to identify genetic variants that confer susceptibility to atherothrombotic cerebral infarction among individuals with metabolic syndrome in order to allow prediction of genetic risk for this condition. The study population comprised 1284 unrelated Japanese individuals with metabolic syndrome, including 313 subjects with atherothrombotic cerebral infarction and 971 controls. The genotypes for 296 polymorphisms of 202 candidate genes were determined with a method that combines the polymerase chain reaction and sequence-specific oligonucleotide probes with suspension array technology. The Chi-square test, multivariable logistic regression analysis with adjustment for age, sex, body mass index, and the prevalence of hypertension, hypercholesterolemia, and diabetes mellitus, as well as a stepwise forward selection procedure revealed that the 2445G→A (Ala54Thr) polymorphism (rs1799883) of *FABP2*, the -108/3G→4G polymorphism of *IPF1* (S82168), the A→G (Thr94Ala) polymorphism (rs2241883) of *FABP1*, the G→A (Asp2213Asn) polymorphism (rs529038) of *ROS1*, the -11377C→G polymorphism (rs266729) of *ADIPOQ*, the 162A→C polymorphism (rs4769055) of *ALOX5AP*, the -786T→C polymorphism (rs2070744) of *NOS3*, and the 3279C→T polymorphism (rs7291467) of *LGALS2* were associated ($P<0.05$) with the prevalence of atherothrombotic cerebral infarction. Among these polymorphisms, the 2445G→A (Ala54Thr) polymorphism of *FABP2* was most significantly associated with this condition. Our results suggest that *FABP2*, *IPF1*, *FABP1*, *ROS1*, *ADIPOQ*, *ALOX5AP*, *NOS3*, and *LGALS2* are susceptibility loci for atherothrombotic cerebral infarction among Japanese individuals with metabolic syndrome. Genotypes for these polymorphisms, especially for the 2445G→A (Ala54Thr) polymorphism of *FABP2*, may prove informative for the prediction of genetic risk for atherothrombotic cerebral infarction among such individuals.

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Key words: atherothrombotic cerebral infarction, genetics, ischemic stroke, metabolic syndrome, polymorphism

Introduction

Metabolic syndrome is defined by a clustering of abdominal obesity, an increased serum concentration of triglycerides, a decreased serum concentration of high density lipoprotein (HDL)-cholesterol, high blood pressure, and an increased fasting blood glucose level (1). Although metabolic syndrome has been recognized as a risk factor for atherosclerotic diseases such as coronary heart disease (2,3) and ischemic stroke (4-8), genetic risk for ischemic stroke in individuals with metabolic syndrome has remained uncharacterized. Given that stroke is the leading cause of severe disability and the third leading cause of death, after heart disease and cancer, in western countries and Japan (9), the identification of biomarkers for stroke risk is important both for risk prediction and for intervention to avert future events.

In light of the above, we performed an association study for 296 candidate gene polymorphisms and atherothrombotic cerebral infarction in 1284 Japanese individuals with metabolic syndrome. The aim of the present study was to identify genetic variants that confer susceptibility to atherothrombotic

cerebral infarction among individuals with metabolic syndrome in order to allow prediction of genetic risk for this condition.

Materials and methods

Study population. The study population comprised 1284 unrelated Japanese individuals who visited outpatient clinics of, or were admitted to, one of the participating hospitals (Gifu Prefectural General Medical Center and Gifu Prefectural Tajimi Hospital in Gifu Prefecture, Japan; and Hirosaki University Hospital, Reimeikyo Rehabilitation Hospital, and Hirosaki Stroke Center in Aomori Prefecture, Japan) between October 2002 and June 2007 because of various symptoms or for an annual health checkup, or who were recruited to a population-based prospective cohort study of aging and age-related diseases in Gunma Prefecture, Japan. Diagnosis of metabolic syndrome was based on a modified version of the definition of metabolic syndrome proposed by the American Heart Association and the US National Heart, Lung, and Blood Institute (1). In this modified version, which was also used in the West of Scotland Coronary Prevention Study (10) and the Women's Health Study (11), body mass index (BMI) replaces waist circumference. On the basis of the recent recognition of a need to revise BMI criteria for obesity in Japanese and other Asian populations (12), we set the cutoff point for obesity as a BMI of ≥25 kg/m^2. A total of 1284 subjects with metabolic syndrome had thus three or more of the following five components: i) a BMI of ≥25 kg/m^2; ii) a serum concentration of triglycerides of ≥1.65 mmol/l (150 mg/dl) or drug treatment for elevated triglycerides; iii) a serum concentration of HDL-cholesterol of <1.04 mmol/l (40 mg/dl) for men or <1.30 mmol/l (50 mg/dl) for women, or drug treatment for reduced HDL-cholesterol; iv) a systolic blood pressure of ≥130 mmHg or diastolic blood pressure of ≥85 mmHg, or drug treatment for hypertension; and v) a fasting plasma glucose level of ≥5.50 mmol/l (100 mg/dl) or drug treatment for elevated glucose.

Among the 1284 subjects with metabolic syndrome, 313 individuals (193 men, 120 women) had atherothrombotic cerebral infarction. The diagnosis of ischemic stroke was based on the occurrence of a new and abrupt focal neurological deficit, with neurological symptoms and signs persisting for >24 h; it was confirmed by positive findings in computed tomography or magnetic resonance imaging (or both) of the head. The type of stroke was determined according to the Classification of Cerebrovascular Diseases III (13). Individuals with cardiogenic embolic infarction, lacunar infarction alone, transient ischemic attack, moyamoya disease, or cerebral venous sinus thrombosis were excluded from the study, as were those with atrial fibrillation in the absence or presence of valvular heart disease. The 971 control subjects (473 men, 498 women) had metabolic syndrome but had no history of ischemic or hemorrhagic stroke or other cerebral diseases; of coronary heart disease, peripheral arterial occlusive disease, or other atherosclerotic diseases; or of other thrombotic, embolic, or hemorrhagic disorders. The study protocol complied with the Declaration of Helsinki and was approved by the Committees on the Ethics of Human Research of Mie University Graduate School of Medicine, Hirosaki University

Table I. Primers, probes, and other PCR conditions for genotyping of polymorphisms examined in the study.

Gene	Polymorphism	Sense primer	Antisense primer	Probe 1	Probe 2	AT	Cy
NOS3	-786T→C	CCACCTGCATTCTGGGAACTG	CTGTCATTCAGTGACGCACGCT	CAGGGTCAGCCAGCCAGGGA	CTCTTCCCTGGCCGGCTGAC	60	50
FABP2	2445G→A (Ala54Thr)	AGCTGACAATTACACAAGAAGGAA	GTTGTAATTAAAGGTGACACCAAG	AATGTTTCGAAAAGCGCTTGATT	TCAAAGAATCAAGCACTTTTCGA	60	50
ADRB3	190T→C (Trp64Arg)	GGGAGGCAACCTGCTGGTCAT	GCTGCGGCCAGCGAAGTCA	GTCTCGGAGTCCAGGCGAT	TCTCGGAGTCCGGGCGAT	60	50
ALOX5AP	162A→C	AGGCAATGTTGTCCTGTTGGCCATCG	GCCTGACTTCCAAACAACCATCAAAG	AAGGAAAGCCCTTCAATCAGG	CTTCCCTGAGTGAAGGGC	60	50
HMOX1	-413T→A	GGGGTTGCTAAGTTCCTGATGT	GGCGTCCCAGAAGGTTCCAG	CCACCAGGCTATTGCTCTGA	TGCTCAGAGCAAAAGCCTGGT	60	50
FABP1	A→G (Thr94Ala)	TCTCTGTTCCCTGCAGACAGTGG	GTCGCCGTTGAGTTCGGTCA	AACTGGTGACAACTTTCAA	AACTGtTGACAGCTTTCAAAAACA	60	50
THBS2	3949T→G	AACCCAAGTGCCTTCAGAGGAT	CTCCACATAAAGTCTCATATATCAC	GATGTTCATCTCTGAGTTCCA	GATGTTCATCTCTGCGTTCCA	60	50
LTA4H	A→G (rs2660845)	CTTCCTGTGGACTTCATAGTGTCTACC	CTGACGCAGGGTGTATCGAGCC	CTACCACTGGCCCCACGGTGCT	AAGCTGCAGAGCCCCCGCGGTCCA	60	50
LGALS2	3279C→T	AGGGAGCCATCTCCTGATGCT	GCCACACAGACACTCACAGAC	CGCACACACACGTCTAACA	CGCACACACACATCTAACAC	60	50
LIPC	-250G→A	CAGCCACGTGGAAGCCACCT	TCGATTTACAGAAGTGCTTCTTATC	CCAAATTAATCAATTTAAAGCTACT	GTTCCAAATTAATCAACTTAAAGCT	60	50
ADIPOQ	-11377C→G	TAATTCATCAGAATGTGTGGCTTG	TTAGGCTTGAAGTGGCAACATTC	GCTCAGATCCTGCCCTTCAAA	GTTTTGTTTTTGAAGCGCAGGAT	60	50
LTA4H	A→G (rs2540482)	TTATAATATACTGTGAATAACTGGTTA	CCTTCAAGGTCTTACTAACATTGCC	AAAGCTTACATTCATCTTTTAATCCCT	CAAGGGATTAAAAGATGAACGTAAGC	60	50
ADIPOR2	795G→A	CATCTGTGTGCTGGGCATTG	CCCCGTGCTCTTACCTGCTC	TAGTCTCCCAGTGGGACAT	TAGTCTCCCAATGGGACATG	60	50
IPF1	-108/3G→4G	TGGCTGTGGGTTCCCTCTGAG	GATTTGGCACTGTGTGGCGTTC	CGAGCAGGGGTGGCGCC	GGCGCCACCCTGCTCGCT	60	50
LIPC	-514C→T	TGGCAAGGGCATCTTTGCTTC	TGGGTTCAGTGAAATTGGTGATGC	TTCACCCCCGTGTCAAAGG	TTCACCCCCATGTCAAAGG	60	50
ROS1	G→A (Asp2213Asn)	TGGGCTCAAGAACCCGACCAA	TGACTCCACTGTTGTTTGCTTCAT	AACTGAAGTTGGTTCCTGAATT	AACTGAAGTTGGTTCTGAATTC	60	50
ROS1	G→C (Cys2229Ser)	TCAGAACCAACTTCAGTTATTCAGAA	AGCTTTCATTTATGACTCCACTGTTG	GCATTTATTAGTGCAGAGATGA	GCATTTATTAGTCCAGAGATGAAGC	60	50

Oligonucleotide sequences are 5'→3'. AT, annealing temperature (°C); Cy, cycles.

Table II. Characteristics of subjects with atherothrombotic cerebral infarction (ACI) and controls among individuals with metabolic syndrome.

Characteristic	ACI	Controls	P
No. of subjects	313	971	
Age (years)	67.0±9.7	68.2±9.2	0.0508
Sex (male/female, %)	61.7/38.3	48.7/51.3	<0.0001
BMI (kg/m^2)	24.5±3.5	25.3±3.2	0.0001
Current or former smoker (%)	23.7	24.1	0.8886
Hypertension (%)	87.2	63.8	<0.0001
Systolic blood pressure (mmHg)	153±27	144±20	<0.0001
Diastolic blood pressure (mmHg)	84±17	82±12	0.0022
Hypercholesterolemia (%)	53.3	36.8	<0.0001
Serum total cholesterol (mmol/l)	5.35±1.11	5.26±0.94	0.1422
Serum triglycerides (mmol/l)	1.97±1.10	2.20±1.34	0.0057
Serum HDL-cholesterol (mmol/l)	1.18±0.35	1.26±0.32	0.0003
Diabetes mellitus (%)	57.2	25.5	<0.0001
Fasting plasma glucose (mmol/l)	7.66±2.94	7.40±3.30	0.2184
Glycosylated hemoglobin (%)	6.27±1.50	5.84±1.48	0.0003

Quantitative data are means ± SD. Smoker: smoking ≥10 cigarettes daily. Hypertension: systolic blood pressure of ≥140 mmHg or diastolic blood pressure of ≥90 mmHg (or both), or taking antihypertensive medication. Hypercholesterolemia: serum total cholesterol of ≥5.72 mmol/l (220 mg/dl) or taking lipid-lowering medication. Diabetes mellitus: fasting blood glucose of ≥6.93 mmol/l (126 mg/dl) or glycosylated hemoglobin content (hemoglobin A1c) of ≥6.5% (or both), or taking antidiabetes medication.

Graduate School of Medicine, Gifu International Institute of Biotechnology, Tokyo Metropolitan Institute of Gerontology, and participating hospitals. Written informed consent was obtained from each participant.

Selection and genotyping of polymorphisms. Our aim was to identify genetic variants associated with atherothrombotic cerebral infarction among Japanese individuals with metabolic syndrome in a case-control association study by examining the relations of one to five polymorphisms of each candidate gene with this condition. With the use of public databases, including PubMed (NCBI) and Online Mendelian Inheritance in Man (NCBI), we selected 202 candidate genes that have been characterized and suggested to be associated with atherothrombotic cerebral infarction. On the basis of published studies or by searching PubMed and single nucleotide polymorphism (SNP) databases [dbSNP (NCBI) and Japanese SNP database (JSNP)], we further selected 296 polymorphisms of these genes, most located in the promoter region or exons, that might be expected to result in changes in the function or expression of the encoded protein (14,15). Wild-type and variant alleles of the polymorphisms were determined from the original sources.

Venous blood (7 ml) was collected into tubes containing 50 mmol/l EDTA (disodium salt), and genomic DNA was isolated with a kit (Genomix; Talent, Trieste, Italy). Genotypes of the 296 polymorphisms were determined at G&G Science (Fukushima, Japan) by a method that combines the polymerase chain reaction (PCR) and sequence-specific oligonucleotide probes with suspension array technology (Luminex, Austin, TX, USA). Primers, probes, and other PCR conditions for genotyping polymorphisms found to be related (P<0.05) to atherothrombotic cerebral infarction by the Chi-square test are shown in Table I. Detailed genotyping methodology was described previously (16).

Statistical analysis. Quantitative data were compared between subjects with atherothrombotic cerebral infarction and controls by the unpaired Student's t-test. Categorical data were compared by the Chi-square test. Allele frequencies were estimated by the gene counting method, and the Chi-square test was used to identify departure from Hardy-Weinberg equilibrium. In the initial screen, genotype distributions for each polymorphism were compared between subjects with atherothrombotic cerebral infarction and controls with the Chi-square test. Polymorphisms with a P-value of <0.05 were further examined in a more rigorous evaluation of association by multivariable logistic regression analysis with adjustment for covariates that differed significantly between subjects with atherothrombotic cerebral infarction and controls. Given that the difference in age was marginally significant, it was included in covariates. Multivariable logistic regression analysis was thus performed with atherothrombotic cerebral infarction as a dependent variable and independent variables including age, sex (0, woman; 1, man), BMI, metabolic variables (0, no history of hypertension, diabetes mellitus, or hypercholesterolemia; 1, positive history), and genotype of each polymorphism, and the P-value, odds ratio, and 95% confidence interval were calculated. Genotypes were assessed according to dominant,

Table III. Genotype distributions of polymorphisms related (P<0.05) to atherothrombotic cerebral infarction (ACI) among individuals with metabolic syndrome as determined by the Chi-square test.

Gene symbol	Polymorphism	dbSNP[a]	ACI		Controls		P
NOS3	-786T→C	rs2070744					0.0025
	TT		256	(82.3)	748	(77.0)	
	TC		46	(14.8)	213	(21.9)	
	CC		9	(2.9)	10	(1.0)	
FABP2	2445G→A (Ala54Thr)	rs1799883					0.0028
	GG		114	(36.7)	448	(46.1)	
	GA		140	(45.0)	405	(41.7)	
	AA		57	(18.3)	118	(12.2)	
ADRB3	190T→C (Trp64Arg)	rs4994					0.0104
	TT		215	(69.1)	627	(64.6)	
	TC		78	(25.1)	314	(32.3)	
	CC		18	(5.8)	30	(3.1)	
ALOX5AP	162A→C	rs4769055					0.0104
	AA		93	(29.9)	231	(23.8)	
	AC		159	(51.1)	483	(49.7)	
	CC		59	(19.0)	257	(26.5)	
HMOX1	-413T→A	rs2071746					0.0105
	TT		69	(22.2)	292	(30.1)	
	TA		167	(53.7)	438	(45.1)	
	AA		75	(24.1)	241	(24.8)	
FABP1	A→G (Thr94Ala)	rs2241883					0.0129
	AA		162	(52.1)	581	(59.9)	
	AG		137	(44.1)	338	(34.9)	
	GG		12	(3.9)	51	(5.3)	
THBS2	3949T→G (3'-UTR)	rs8089					0.0133
	TT		247	(79.4)	832	(85.7)	
	TG		60	(19.3)	136	(14.0)	
	GG		4	(1.3)	3	(0.3)	
LTA4H	A→G	rs2660845					0.0157
	AA		60	(19.3)	150	(15.5)	
	AG		125	(40.2)	480	(49.4)	
	GG		126	(40.5)	341	(35.1)	
LGALS2	3279C→T (intron 1)	rs7291467					0.0181
	CC		153	(49.2)	426	(43.9)	
	CT		137	(44.1)	429	(44.2)	
	TT		21	(6.8)	116	(12.0)	
LIPC	-250G→A	rs2070895					0.0187
	GG		91	(29.3)	246	(25.3)	
	GA		127	(40.8)	485	(50.0)	
	AA		93	(29.9)	240	(24.7)	
ADIPOQ	-11377C→G	rs266729					0.0207
	CC		163	(52.4)	575	(59.2)	
	CG		120	(38.6)	346	(35.6)	
	GG		28	(9.0)	50	(5.2)	
LTA4H	A→G	rs2540482					0.0214
	AA		58	(18.0)	145	(15.1)	
	AG		124	(39.9)	470	(48.9)	
	GG		131	(42.1)	347	(36.1)	
ADIPOR2	795G→A	rs16928751					0.0255
	GG		303	(97.4)	962	(99.2)	
	GA		8	(2.6)	8	(0.8)	
	AA		0	(0)	0	(0)	

Table III. Continued.

Gene symbol	Polymorphism	dbSNP[a]	ACI	Controls	P
IPF1	-108/3G→4G	S82168			0.0280
	3G3G		96 (30.9)	226 (23.3)	
	3G4G		134 (43.1)	475 (48.9)	
	4G4G		81 (26.1)	270 (27.8)	
LIPC	-514C→T	rs1800588			0.0296
	CC		91 (29.3)	239 (24.6)	
	CT		130 (41.8)	489 (50.4)	
	TT		90 (28.9)	242 (25.0)	
ROS1	G→A (Asp2213Asn)	rs529038			0.0311
	GG		225 (72.4)	709 (73.0)	
	GA		74 (23.8)	249 (25.6)	
	AA		12 (3.9)	13 (1.3)	
ROS1	G→C (Cys2229Ser)	rs619203			0.0375
	GG		10 (4.1)	12 (1.3)	
	GC		50 (20.3)	196 (21.5)	
	CC		186 (75.6)	702 (77.1)	

Numbers in parentheses are percentages. [a]In instances in which rs numbers in dbSNP were not detected, NCBI GenBank accession numbers are shown.

recessive, and two additive (additive 1 and 2) genetic models. Each genetic model comprised two groups: the combined group of variant homozygotes and heterozygotes versus wild-type homozygotes for the dominant model; variant homozygotes versus the combined group of wild-type homozygotes and heterozygotes for the recessive model; heterozygotes versus wild-type homozygotes for the additive 1 model; and variant homozygotes versus wild-type homozygotes for the additive 2 model. We also performed a stepwise forward selection procedure to examine the effects of genotypes as well as of other covariates on atherothrombotic cerebral infarction. The P-levels for inclusion in and exclusion from the model were 0.25 and 0.1, respectively. In the stepwise forward selection procedure, each genotype was examined according to a dominant or recessive model on the basis of statistical significance in the multivariable logistic regression analysis. For all statistical analysis, a P-value of <0.05 was considered significant. Statistical significance was examined by two-sided tests, and statistical analysis was performed with JMP version 6.0 software (SAS Institute, Cary, NC, USA).

Results

The characteristics of the 1284 study subjects are shown in Table II. The frequency of male subjects, the prevalence of hypertension, hypercholesterolemia, and diabetes mellitus, systolic and diastolic blood pressure, and the percentage of glycosylated hemoglobin were greater, whereas BMI and the serum concentrations of triglycerides and HDL-cholesterol were lower, in subjects with atherothrombotic cerebral infarction than in controls.

Comparisons of genotype distributions with the Chi-square test revealed that the -786T→C polymorphism (rs2070744) of *NOS3*, the 2445G→A (Ala54Thr) polymorphism (rs1799883) of *FABP2*, the 190T→C (Trp64Arg) polymorphism (rs4994) of *ADRB3*, the 162A→C polymorphism (rs4769055) of *ALOX5AP*, the -413T→A polymorphism (rs2071746) of *HMOX1*, the A→G (Thr94Ala) polymorphism (rs2241883) of

Table IV. Hardy-Weinberg P-values in subjects with atherothrombotic cerebral infarction (ACI) and controls.

Gene	Polymorphism	ACI	Controls
NOS3	-786T→C	0.0014[a]	0.2905
FABP2	2445G→A (Ala54Thr)	0.2763	0.0892
ADRB3	190T→C (Trp64Arg)	0.0075[a]	0.2552
ALOX5AP	162A→C	0.6169	0.9410
HMOX1	-413T→A	0.2310	0.0037[a]
FABP1	A→G (Thr94Ala)	0.0137[a]	0.9121
THBS2	3949T→G (3'-UTR)	0.8994	0.4232
LTA4H	A→G (rs2660845)	0.0075[a]	0.4121
LGALS2	3279C→T (intron 1)	0.2390	0.6673
LIPC	-250G→A	0.0018[a]	0.9735
ADIPOQ	-11377C→G	0.4675	0.8969
LTA4H	A→G (rs2540482)	0.0098[a]	0.5345
ADIPOR2	795G→A	0.0451[a]	0.0002[a]
IPF1	-108/3G→4G	0.0221[a]	0.5845
LIPC	-514C→T	0.0054[a]	0.8470
ROS1	G→A (Asp2213Asn)	0.1064	0.1149
ROS1	G→C (Cys2229Ser)	0.0182[a]	0.8037

[a]P<0.05.

Table V. Multivariable logistic regression analysis of polymorphisms related to atherothrombotic cerebral infarction by the Chi-square test for individuals with metabolic syndrome.

Gene	Polymorphism	Dominant		Recessive		Additive 1		Additive 2	
		P	OR (95% CI)	P	OR (95% CI)	P	OR (95% CI)	P	OR (95% CI)
NOS3	-786T→C	0.0851		0.0619		0.0253	0.65 (0.44-0.94)	0.0878	
FABP2	2445G→A (Ala54Thr)	0.0031	1.55 (1.16-2.07)	0.0048	1.75 (1.18-2.57)	0.0316	1.40 (1.03-1.91)	0.0007	2.08 (1.36-3.17)
ADRB3	190T→C (Trp64Arg)	0.2129		0.1318		0.0798		0.2096	
ALOX5AP	162A→C	0.0305	0.71 (0.52-0.97)	0.0028	0.59 (0.41-0.83)	0.2281		0.0015	0.51 (0.34-0.77)
FABP1	A→G (Thr94Ala)	0.0227	1.39 (1.05-1.84)	0.1291		0.0056	1.51 (1.13-2.02)	0.2983	
THBS2	3949T→G (3'-UTR)	0.1316		0.0206	6.49 (1.31-35.30)	0.2721		0.0187	6.68 (1.35-36.31)
LTA4H	A→G (rs2660845)	0.4756		0.2439		0.2234		0.9804	
LGALS2	3279C→T (intron 1)	0.2654		0.0209	0.54 (0.31-0.89)	0.6975		0.0197	0.53 (0.30-0.89)
LIPC	-250G→A	0.2322		0.1816		0.0667		0.9330	
ADIPOQ	-11377C→G	0.0867		0.0062	2.14 (1.23-3.68)	0.3341		0.0040	2.27 (1.29-3.95)
LTA4H	A →G (rs2540482)	0.7186		0.1928		0.3718		0.7866	
IPF1	-108/3G→4G	0.0065	0.65 (0.47-0.89)	0.6625		0.0062	0.62 (0.44-0.87)	0.0550	
LIPC	-514C→T	0.1194		0.4117		0.0459	0.71 (0.50-0.99)	0.6532	
ROS1	G→A (Asp2213Asn)	0.3800		0.0243	2.78 (1.13-6.83)	0.7660		0.0233	2.82 (1.14-6.94)
ROS1	G→C (Cys2229Ser)	0.0142	0.28 (0.10-0.78)	0.2284		0.0285	0.31 (0.11-0.89)	0.0129	0.28 (0.10-0.77)

OR, odds ratio; CI, confidence interval. Multivariable logistic regression analysis was performed with adjustment for age, sex, BMI, and the prevalence of hypertension, hypercholesterolemia, and diabetes mellitus.

Table VI. Effects of genotypes and other characteristics on atherothrombotic cerebral infarction among individuals with metabolic syndrome determined by a stepwise forward selection procedure (P<0.05).

Variable	P	R^2
Diabetes mellitus	<0.0001	0.0732
Hypertension	<0.0001	0.0419
BMI	<0.0001	0.0140
Hypercholesterolemia	0.0004	0.0107
Sex	0.0012	0.0089
FABP2 (*GA* + *AA* versus *GG*)	0.0037	0.0072
IPF1 (*3G4G* + *4G4G* versus *3G3G*)	0.0051	0.0067
FABP1 (*AG* + *GG* versus *AA*)	0.0063	0.0063
ROS1 (rs529038) (*AA* versus *GG* + *GA*)	0.0080	0.0060
ADIPOQ (*GG* versus *CC* + *CG*)	0.0082	0.0059
ALOX5AP (*CC* versus *AA* + *AC*)	0.0149	0.0050
NOS3 (*CC* versus *TT* + *TC*)	0.0237	0.0044
LGALS2 (*TT* versus *CC* + *CT*)	0.0405	0.0036

R^2, contribution rate.

FABP1, the 3949T→G polymorphism (rs8089) of *THBS2*, the A→G polymorphism (rs2660845) of *LTA4H*, the 3279C→T polymorphism (rs7291467) of *LGALS2*, the -250G→A polymorphism (rs2070895) of *LIPC*, the -11377C→G polymorphism (rs266729) of *ADIPOQ*, the A→G polymorphism (rs2540482) of *LTA4H*, the 795G→A polymorphism (rs16928751) of *ADIPOR2*, the -108/3G→4G polymorphism of *IPF1* (S82168), the -514C→T polymorphism (rs1800588) of *LIPC*, the G→A (Asp2213Asn) polymorphism (rs529038) of *ROS1*, and the G→C (Cys2229Ser) polymorphism (rs619203) of *ROS1* were related (P<0.05) to atherothrombotic cerebral infarction (Table III). The genotype distributions of these 17 polymorphisms in subjects with atherothrombotic cerebral infarction and in controls are also shown in Table III. In control subjects, the genotype distributions of these polymorphisms with the exception of those of *HMOX1* and *ADIPOR2* were in Hardy-Weinberg equilibrium (Table IV); the polymorphisms of *HMOX1* and *ADIPOR2* were therefore excluded from subsequent analysis.

Multivariable logistic regression analysis with adjustment for age, sex, BMI, and the prevalence of hypertension, diabetes mellitus, and hypercholesterolemia revealed that the -786T→C polymorphism of *NOS3* (additive 1 model), the 2445G→A (Ala54Thr) polymorphism of *FABP2* (dominant, recessive, and additive 1 and 2 models), the 162A→C polymorphism of *ALOX5AP* (dominant, recessive, and additive 2 models), the A→G (Thr94Ala) polymorphism of *FABP1* (dominant and additive 1 models), the 3949T→G polymorphism of *THBS2* (recessive and additive 2 models), the 3279C→T polymorphism of *LGALS2* (recessive and additive 2 models), the -11377C→G polymorphism of *ADIPOQ* (recessive and additive 2 models), the -108/3G→4G polymorphism of *IPF1* (dominant and additive 1 models), the -514C→T polymorphism of *LIPC* (additive 1 model), the G→A (Asp2213Asn) polymorphism of *ROS1* (recessive and additive 2 models), and the G→C (Cys2229Ser) polymorphism of *ROS1* (dominant and additive 1 and 2 models) were associated (P<0.05) with the prevalence of atherothrombotic cerebral infarction (Table V). The variant *A* allele of *FABP2*, *G* allele of *FABP1*, *G* allele of *THBS2*, *G* allele of *ADIPOQ*, and *A* allele of the G→A (Asp2213Asn) polymorphism of *ROS1* were risk factors for atherothrombotic cerebral

infarction, whereas the variant *C* allele of *NOS3*, *C* allele of *ALOX5AP*, *T* allele of *LGALS2*, 4*G* allele of *IPF1*, *T* allele of the -514C→T polymorphism of *LIPC*, and *C* allele of the G→C (Cys2229Ser) polymorphism of *ROS1* were protective against this condition.

Finally, we performed a stepwise forward selection procedure to examine the effects of genotypes for the 11 polymorphisms associated with atherothrombotic cerebral infarction by multivariable logistic regression analysis as well as of age, sex, BMI, and the prevalence of hypertension, diabetes mellitus, and hypercholesterolemia on atherothrombotic cerebral infarction (Table VI). Diabetes mellitus, hypertension, BMI, hypercholesterolemia, sex, *FABP2* genotype (dominant model), *IPF1* genotype (dominant model), *FABP1* genotype (dominant model), *ROS1* genotype (rs529038, recessive model), *ADIPOQ* genotype (recessive model), *ALOX5AP* genotype (recessive model), *NOS3* genotype (recessive model), and *LGALS2* genotype (recessive model), in descending order of statistical significance, were independent ($P<0.05$) determinants of atherothrombotic cerebral infarction.

Discussion

We examined the possible relations of 296 polymorphisms in 202 candidate genes to the prevalence of atherothrombotic cerebral infarction in 1284 Japanese individuals with metabolic syndrome. Our association study with three steps of analysis (Chi-square test, multivariable logistic regression analysis, and stepwise forward selection procedure) revealed that the 2445G→A (Ala54Thr) polymorphism of *FABP2*, the -108/3G→4G polymorphism of *IPF1*, the A→G (Thr94Ala) polymorphism of *FABP1*, the G→A (Asp2213Asn) polymorphism of *ROS1*, the -11377C→G polymorphism of *ADIPOQ*, the 162A→C polymorphism of *ALOX5AP*, the -786T→C polymorphism of *NOS3*, and the 3279C→T polymorphism of *LGALS2* were associated with the prevalence of atherothrombotic cerebral infarction. Among these polymorphisms, the 2445G→A (Ala54Thr) polymorphism of *FABP2* was most significantly associated with this condition.

Fatty acid-binding protein 2 (FABP2) is an intracellular protein that is expressed only in the columnar absorptive epithelial cells of the small intestine. It contains a single ligand site that has a high affinity for saturated and unsaturated fatty acids, and it contributes to the absorption and intracellular transport of long-chain fatty acids (17). The product of the *A* allele of the 2445G→A (Ala54Thr) polymorphism of *FABP2* possesses a greater affinity for long-chain fatty acids *in vitro* than does that of the *G* allele (18). In addition, individuals with the *A* allele of this polymorphism were found to be more insulin resistant than were those with the *G* allele (18,19). The *A* allele was also shown to be associated with higher plasma levels of low density lipoprotein-cholesterol (20) or with dyslipidemia (high plasma concentration of triglycerides and low concentration of HDL-cholesterol) (21). In addition, the *A* allele of the 2445G→A (Ala54Thr) polymorphism was previously associated with a parental history of stroke in the Swedish population (22). Moreover, it was associated with a 2- to 3.5-fold increase in cardiovascular risk in dyslipidemic men with diabetes compared with their dyslipidemic nondiabetic counterparts; for nonfatal myocardial infarction, stroke, or death from coronary heart disease, the corresponding hazard ratio was 3.0, whereas for stroke alone it was 3.5 (23). Our results show that the 2445G→A (Ala54Thr) polymorphism of *FABP2* was significantly associated with atherothrombotic cerebral infarction in individuals with metabolic syndrome, with the *A* (Thr) allele representing a risk factor for this condition. The effects of this polymorphism on both insulin resistance and lipid metabolism may account for its association with atherothrombotic cerebral infarction.

Among the seven polymorphisms of *IPF1*, *FABP1*, *ROS1* (rs529038), *ADIPOQ*, *ALOX5AP*, *NOS3*, and *LGALS2* also associated with atherothrombotic cerebral infarction in individuals with metabolic syndrome, the 162A→C polymorphism of *ALOX5AP* and the -786T→C polymorphism of *NOS3* have previously been associated with ischemic stroke (24,25). The -108/3G→4G polymorphism of *IPF1*, the G→A (Asp2213Asn) polymorphism of *ROS1*, the -11377C→G polymorphism of *ADIPOQ*, and the 3279C→T polymorphism of *LGALS2* were found not to be associated with ischemic stroke, but with myocardial infarction (26-30). The remaining A→G (Thr94Ala) polymorphism of *FABP1* has not been reported to be associated with ischemic stroke or myocardial infarction.

Given the multiple comparisons of genotypes with atherothrombotic cerebral infarction in the present study, it is not possible to exclude completely potential statistical errors such as false positives. It is also possible that one or more of the polymorphisms associated with this type of stroke in the present study are in linkage disequilibrium with other polymorphisms in the same gene or in other nearby genes that are actually responsible for the development of this condition. In addition, the functional relevance of the identified polymorphisms to gene transcription or to protein structure or function was not determined in the present study.

In conclusion, our present results suggest that *FABP2*, *IPF1*, *FABP1*, *ROS1*, *ADIPOQ*, *ALOX5AP*, *NOS3*, and *LGALS2* are susceptibility loci for atherothrombotic cerebral infarction among Japanese individuals with metabolic syndrome. Genotypes for these polymorphisms, especially for the 2445G→A (Ala54Thr) polymorphism of *FABP2*, may prove informative for assessment of genetic risk for atherothrombotic cerebral infarction among individuals with metabolic syndrome. Validation of our findings will require their replication with independent subject panels.

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Galectin-2 3279TT variant protects against the lymphotoxin-α 252GG genotype associated ischaemic stroke

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ABSTRACT

Objective: The galectin-2 protein is presumed to play a regulatory role in the intracellular trafficking of the lymphotoxin-α (*LTA*) cytokine. *LTA* is a pro-inflammatory factor, its 252GG homozygote variant is considered as a susceptibility factor for arteriosclerosis and cardiovascular diseases. By contrast, the galectin-2-encoding gene *LGALS2* 3279TT homozygote variant has been demonstrated to exert protection against myocardial infarction by reducing the transcriptional level of galectin-2, thereby leading to a reduced extracellular secretion of *LTA*.
Methods: In the present study, we examined whether the *LGALS2* 3279TT homozygote variant alone can influence the prevalence of ischaemic stroke, and whether it can interact somehow with the disadvantageous *LTA* 252GG homozygote variant. Genetic and clinical data of 385 ischemic stroke patients and 303 stroke and neuroimaging alteration-free controls were analysed.
Results: The combination of the *LGALS2* 3279TT and *LTA* 252GG homozygote was significantly less frequent in the ischemic stroke group (1.56%) than in the controls (5.94%, $p < 0.00187$; overall stroke group: crude OR: 0.25, 95% CI: 0.1–0.64; adjusted OR: 0.03, 95% CI: 0.025–0.71).
Conclusions: This finding suggests a gene–gene interaction.

1. Introduction

Lymphotoxin-alpha (*LTA*), a cytokine affecting pro-inflammatory processes, is a factor promoting the development of atherosclerosis [1–8]. The *LTA* gene has been implicated in the pathomechanism of ischemic stroke and myocardial infarction. the *LTA* 252GG homozygote, which naturally coexists with the 804AA homozygote, has also been demonstrated to contribute to myocardial infarction or large-vessel-associated ischemic stroke [9–14]. In consistence with these observations, a recent article demonstrated that the *LTA* 252GG homozygote variant is associated with an increased intima-media thickness in the carotid arteries [15]. In contrast, another study reported that the *LTA* 252GG homozygote can be protective against certain types of ischemic stroke [16]. The contradictory findings may stem from the fact that some other genetic variant can modify the effects of the *LTA* 252GG homozygote or that ischemic stroke is a heterogeneous entity caused by different pathomechanisms.

LTA binds to the galectic-2 transport protein, which is implicated as playing an important role in the intracellular trafficking of *LTA* [17]. Galectin-2-encoding *LGALS2* gene C3279T single nucleotide polymorphism is presumed to reduce the transcriptional level of galectin-2, and can thereby protect against myocardial infarction [17]. In this context, the aim of our present study was to examine whether the potentially protective *LGALS2* 3279TT homozygote can influence the unfavourable effects of the *LTA* 252GG homozygote in different types of ischemic stroke.

2. Methods

The study involved 385 Caucasian ischemic stroke patients and 303 stroke and neuroimaging alteration-free Caucasian controls. The controls had negative brain CT or MRI scans, in order to avoid silent brain infarction. The patients and controls underwent clinical scrutiny [18], including an exploration of the medical

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history and the family anamnesis, an evaluation of the vascular risk factors, general physical and neurological examinations, urine analysis, extended laboratory examinations, electrocardiography, extracranial and transcranial Doppler sonography of the brain-supplying arteries, transthoracic and/or transoesophageal echocardiography where appropriate, and MRI examinations. The infarctions were evaluated in the axial and coronal views of the T2-, T1- and proton density-weighted images. All scans were read by an experienced investigator without knowledge of the clinical and laboratory data. The stroke patients were enrolled into one of the following stroke type groups: large-vessel-associated stroke (cortical or cerebellar lesions and/or brainstem infarcts or subcortical hemispheric infarcts greater than 1.5 cm in diameter on the MRIs), small-vessel ischemic stroke (one or more subcortical hemispheric or brainstem infarcts with a diameter of less than 1.5 cm on the MRIs), or a mixed vascular type (one or more lacunar and large-vessel infarcts on the MRIs). The mode of assessment of the clinical results was also reported earlier [18]. The study design was approved in advance by the Local Ethics Committee, and all participants gave their informed consent to the examinations.

The 303 subjects who served as a control group, did not exhibit any brain MRI scan abnormality. They were believed to be healthy and not to suffer from any vascular brain pathology. They were randomly selected from our practice register with the requirement of negative brain MRI scans. We randomly identified a healthy individual from our regional register then we carried out the MRI examination on him. If it did not show any alterations, we potentially enrolled the subject into our control group. Subjects with any kind of previous clinical data suggesting a cerebrovascular or cardiovascular event, such as a transient ischemic attack or angina pectoris, were excluded from the control group.

The clinical data were given as means + S.D. or in percent. The differences between the clinical parameters and different genotypes in the stroke groups and control group were compared using the χ^2 test or Mann–Whitney test. The interaction terms were assessed in two by two tables by the exact Fisher's and the χ^2 tests. Crude and adjusted odds ratios (OR) were also calculated as a measure of the association between the different genotypes and the stroke groups and are interpreted as the relative risk of the disease for the exposed as compared with the unexposed. All statistical calculations were carried out by SYSTAT 10 for Windows statistical package. We calculated formal statistical power for the statistical tests (alpha = 0.05). Due to multiple genetic testing, Bonferroni correction was also performed. In accordance to previous clinical, biochemical and genetic data, only three variables (two genetic factors and their combination pair) were presumed to have biological phenotype effects, therefore Bonferroni correction did have to be carried out for three independent tests.

Table 1
Major clinical and laboratory data on patients and control subjects.

Clinical features	Overall stroke group (n = 385)	Control group (n = 303)
Sex, females/males	222/163	201/102
Age, years	67.4 ± 13.85*	57.4 ± 14.3
BMI, kg/m^2	25.4 ± 1.71*	23.1 ± 1.71
Cholesterol, mM	5.89 ± 1.21*	5.0 ± 0.84
Triglycerides, mM	1.79 ± 0.65*	1.49 ± 0.5
Hypertension	42.9%*	11.9%
Diabetes mellitus	24.2%*	3%
Smokers	31.9%*	7.9%
Drinkers	10.1%*	3%
Ischaemic heart disease	14.3%*	2%

* $p < 0.048$; the overall stroke group was compared with the control group by the χ^2-test or the Mann–Whitney test where appropriate. BMI: body mass index.

Genomic DNA was extracted from peripheral blood anticoagulated with EDTA by a routine salting out method. A PCR/RFLP method for the detection of the *LTA* A252G and C804A variants was used as reported earlier [10]. The C3279T alteration was determined by PCR-RFLP assay. The primers were designed using the GenBank reference sequence AL022315; the sense primer was 5′-AGGAGCTGCAAGGGAGTGT-3′, the antisense 5′-CCAGTGAGGACAGTCCAAAAGG-3′. The PCR conditions were as follows: initial denaturation at 96 °C followed by 35 cycles of 30 s at 96 °C, 30 s at 60 °C, 30 s at 72 °C and a final extension at 72 °C. The amplification was carried out in a final volume of 50 μl containing 200 mM of each dNTP, 1 U of Taq polymerase, 5 μl of reaction buffer (500 mM KCl, 14 mM $MgCl_2$, 10 mM Tris–HCl, pH 9.0), 0.2 mM of each primer and 1 μg extracted DNA. Finally, 10 μl of the 239 bp long amplicon was digested with one U of *TaiI* restriction endonuclease and the digestion products were separated through an ethidium-bromide stained 3% agarose gel. The primers were designed to create an obligatory cleaving site on the amplicon to enable us to check the digestion. Therefore, after digestion 21, 78, 140 bp long fragments were in the samples with CC genotype. In homozygous TT samples 78, 161 bp long products were detected. In heterozygous patients 21, 78, 140, 161 bp fragments were produced.

The distribution of the genotypes met the requirement of the Hardy–Weinberg equilibrium in the controls.

3. Results

The clinical data are summarized in Table 1.

The frequencies of the *LTA* A252G and *LGALS2* C3279T variants and their combinations are shown in Table 2. The *LTA* 252GG homozygote yielded a risk for large-vessel associated ischemic

Table 2
Distribution of the different genotypes amongst the different stroke subtypes and controls.

Genotypes	Large-vessel (n = 106)	Small-vessel (n = 175)	Mixed group (n = 104)	Overall (n = 385)	Controls (n = 303)
LTA AA	41 (38.7%)	69 (39.4%)	40 (38.5%)	150 (39%)	118 (38.9%)
LTA AG	40 (37.7%)	94 (53.7%)	54 (51.9%)	188 (48.8%)	158 (52.1%)
LTA GG	25 (0.0pt††) (23.6%)	12 (6.86%)	10 (9.62%)	47 (12.2%)	27 (8.91%)
LTA G allele frequency	42.5%	33.7%	35.6%	36.6%	35%
LGALS2 CC	12 (11.3%)	26 (14.9%)	13 (12.5%)	51 (13.2%)	45 (14.9%)
LGALS2 CT	53 (50%)	77 (44%)	51 (49%)	181 (47.0%)	136 (44.9%)
LGALS2 TT	41 (38.7%)	72 (41.1%)	40 (38.5%)	153 (39.7%)	122 (40.3%)
LGALS2 T allele frequency	63.7%	63.1%	63%	63.2%	62.7%
LTA GG + *LGALS2* TT	3 (2.83%)	2* (1.14%)	1** (0.96%)	6 (0.0pt†) (1.56%)	18 (5.94%)

The stroke groups were compared with the controls by the χ^2 or Fisher's exact test.

* $p < 0.0116$.

** $p < 0.0378$.

† $p < 0.00187$.

†† $p < 0.000094$.

Table 3
Crude and adjusted odds ratios (OR) at 95% confidence intervals for the *LTA* 252GG and *LGALS2* L3279TT homozygote containing genotype combination in stroke subtypes.

Genotypes	Large-vessel OR	Small-vessel OR	Mixed group OR	Overall OR
Crude odds ratio				
LTA 252GG	3.16†† (1.74–5.74)	0.75 (0.37–1.53)	1.09 (0.51–2.33)	1.42 (0.86–2.34)
LTA 252GG+*LGALS2* 3279TT	0.46 (0.13–1.6)	0.18* (0.04–0.8)	0.15 (0.02–1.17)	0.25† (0.1–0.64)
Adjusted ORs[a]				
LTA 252GG	3.34†† (1.98–5.98)	0.67 (0.45–1.98)	1.1 (0.45–3.1)	1.56 (0.78–2.45)
LTA 252GG+*LGALS2* 3279TT	0.4 (0.1–1.6)	0.2* (0.07–0.8)	0.18 (0.084–1.75)	0.03† (0.025–0.71)

[a] ORs of the risk-associated genotype combination in stroke subtypes from logistic regression models after adjustment for differences in age, sex, serum cholesterol, serum triglycerides, hypertension, diabetes mellitus, smoking, drinking habits and ischaemic heart diseases. OR indicated the relative risk of stroke for the subject carrying the above genotypes compared to the ones not carrying them.

* $p<0.042$.

† $p<0.0044$.

†† $p<0.00023$.

Table 4
Distribution of *LTA* 252GG combined with or without *LGALS2* 3279TT in stroke subtypes and controls.

Genotypes	Large-vessel ($n=25$)	Small-vessel ($n=12$)	Mixed group ($n=10$)	Overall ($n=47$)	Controls ($n=27$)
LTA 252GG without *LGALS2* 3279TT	22 (88%)	10 (83%)	9 (90%)	41 (87%)	9 (33%)
LTA 252GG with *LGALS2* 3279TT	3 (12%)	2 (16.7%)	1 (10%)	6 (12.8%)	18 (66.7%)
p	<0.00098	<0.00097	<0.0009	<0.0009	

Differences between the frequencies of *LTA* 252GG with or without *LGALS2* 3279TT variant in stroke groups and controls were compared with Fisher's exact test.

stroke (23.6% versus the control 8.91%; $p<0.000094$, Crude OR: 3.16, 95% CI: 1.74–5.74). There was no accumulation of any of *LGALS2* C3279T variants either homozygous or heterozygous form in any groups of stroke. By contrast, the homozygous *LTA* 252GG homozygote in combination with the *LGALS2* 3279TT hymozygote occurred less frequently in the overall ischemic stroke group (1.56%, $p<0.00187$), in the mixed vascular type (0.96%, $p<0.0378$) and in the small-vessel ischemic stroke patients (1.14%, $p<0.0116$) than in the controls (5.94%). The other combination patterns of the *LTA* A252G and *LGALS2* C3279T genotypes did not differ between the stroke groups and the control group.

The crude and adjusted ORs are found in Table 3. The *LGALS2* 3279TT homozygote combined with the *LTA* 252GG homozygote yielded protection against ischemic stroke (overall ischemic stroke group: crude OR: 0.25, 95% CI: 0.1–0.64, $p<0.004$; small-vessel stroke group: crude OR: 0.18, 95% CI: 0.04–0.8, $p<0.04$). After logistic regression calculation, the same combination remained a protective factor.

Table 4 presents the frequencies of the *LGALS2* 3279TT homozygote versus other *LGALS2* genotypes in the subgroups of subjects with the *LTA* 252GG homozygote. Among patients with the *LTA* 252GG homozygote, the combination of the *LGALS2* 3279TT homozygote and *LTA* 252GG homozygote occurred significantly less frequently in all stroke subtypes than in the controls (large-vessel and overall: $p<0.00098$; small-vessel and mixed group: $p<0.0009$). The distribution of the *LGALS2* genotypes in stroke cases with or without *LTG* 252GG homozygote is shown in Table 5.

The formal power calculation for the positive interaction term test as regards the overall stroke group proved to be over 85% (alpha = 0.05). After the Bonferroni correction the found associations remained significant for the overall stroke group (Bonferroni threshold significance level = 0.0166).

4. Discussion

The *LTA* 252GG homozygote conferred a significant risk of large-vessel ischaemic stroke, which was expected because most of the study subjects were the same as in our earlier study, and gave similar results [10]. The distribution of the *LGALS2* C3279T variant was the same in the stroke groups and the controls. However, the presence of the *LGALS2* 3279TT homozygote conferred significant protection against ischemic stroke in the presence of the disadvantageous *LTA* 252GG homozygote. The univariate subgroup analysis revealed that this association mainly resulted from the reduced frequency of this combination genotype in the small-vessel-infarct group. This finding can be explained by the fact that the increased number of the *LTA* 252GG homozygote in the large-vessel stroke group, which resulted from it being a risk factor for this type of stroke, has confounded the protective association for the above combination pair in the large-vessel vascular type. The results of Fisher's exact tests that were carried out on the subgroups of subjects with *LTA* 252GG homozygote only, however, have also confirmed the protective role of the combination of the *LTA* 252GG homozygote and *LGALS2* 3279TT homozygote in large-vessel ischemic stroke. Bonferroni correction did not change the basic associations.

The exact explanation of this protective role of the *LGALS2* 3279TT homozygote is not known. However, there are some indications that the level of galectin-2 is of great importance in the secretion of *LTA in vitro* [17]. A higher level of galectin-2 will lead to a higher level of extracellular secretion of *LTA* in a cell medium [17].

Table 5
Distribution of the *LGALS2* genotypes in stroke cases with different *LTA* A252G genotypes.

Genotypes	Stroke cases with LTA GG ($n=47$)	Stroke cases with LTA AG ($n=188$)	Stroke cases with LTA AA ($n=150$)	Stroke cases without LTA GG ($n=338$)
LGALS2 3279CC	14 (29.8%)	24 (12.8%)	13 (8.7%)	37 (10.9%)
LGALS2 3279CT	27 (57.4%)	75 (39.9%)	79 (52.7%)	154 (45.6%)
LGALS2 3279TT	6* (12.8%)	89 (47.3%)	58 (38.6%)	147 (43.5%)

* $p<0.000055$, Differences between the frequencies of *LGALS2* TT with or without *LTA* GG variant in stroke cases were compared by the χ^2.

The presence of the *LGALS2* 3279TT variant, however, results in a nearly 50% lower transcriptional activity of galectin-2 [17]. The *LTA* 252GG homozygote has been reported to cause a 1.5-fold enhancement of the transcriptional activity of *LTA*, which is considered to be in general a pro-inflammatory and atherosclerosis-promoting factor [9]. When these basic data are taken into account, the possibility arises that the protective role of the *LGALS2* 3279T allele against the development of ischemic stroke materializes only in the presence of the disadvantageous *LTA* 252GG homozygote. The most obvious explanation of this appears to be that the reduced level of galectin-2 determined by the 3279TT homozygote will reduce the extracellular secretion of the disadvantageous *LTA* encoded by the 252GG homozygote; and in this way it can counterbalance the pro-inflammatory effects of the *LTA* 252GG homozygote. In the presence of the more favourable *LTA* A252 allele, the importance of this protective role of the galectin-2 level may be lost as regards the development of ischemic stroke, because the basic production of the *LTA* is not high and in this case the level of the galectin-2 transport protein will be indifferent.

The other hypothetical explanation may be that the *LTA* coded by the unfavourable 252GG homozygote binds to galectin-2 with a different strength as compared with the *LTA* coded by the more favourable *LTA* AA252 homozygote; and because of this changed binding feature, the reduced level of galactin-2 determined by the *LGALS2* 3279TT homozygote will change the secretion of *LTA* to a greater extent in the presence of the *LTA* 252GG homozygote than in the presence of the *LTA* AA252 homozygote. It has also been suggested that the different allelic variations of *LTA* have different binding features [9].

The protective effects of *LGALS2* 3279TT homozygote in the presence of the *LTA* 252GG homozygote may also explain why some studies found that the presence of the *LTA* 252GG homozygote is protective against uncategorized ischemic stroke [16].

Limitations of the study: (1) although the statistical power of the tests was sufficient, the present association needs further examination with a greater case-control study, specially for the subtypes of stroke; (2) the different genotypes can affect the survival of patients, thereby distorting the distribution of the genetic factors in the stroke cases; (3) the gene–gene interaction found demands further confirmation in different geographical regions; (4) because of the low number of positive interactions and the relatively high statistical power of the tests, the clinical importance of the present gene-gene association is open to question.

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